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14. ABSTRACT

- The research proposed to examine the ability of inhibition of mitophagy, the mitochondrial-specific form of autophagy, to kill prostate cancer cells. Cancer cells become increasingly dependent on mitophagy as an energy source and the mitochondria themselves become more and more dysfunctional. Therefore, mitophagy may represent a novel target for the prevention of prostate cancer progression. Consequently, the *purpose* of this research was to test whether inhibition of mitophagy can lead to the death of prostate cancer cells. Key mediators of the mitophagic process, specifically Parkin, dynamin-related protein-1 (Drp1), fission-1 (Fis1), and cyclophilin-D (CypD), were genetically disrupted by siRNA. The effects that mitophagy blockade had on mitochondrial function, ROS production and ultimately survival of several prostate cancer cell lines was then examined. We found that on the whole, inhibition of mitophagy caused a compensatory upregulation of general autophagy and that this appeared to be a direct result of increased ROS production. Depletion of CypD actually improved mitochondrial function and ROS production and was protective against chemotherapeutic-induced cell death. In contrast, Fis1 and Parkin knockdown was sufficient to sensitize LNCaP and PC3 cells to the necrotic effects of doxorubicin. Finally, we found that Drp1 knockdown was sufficient to impair mitochondrial function, induce ROS production and reduce cell proliferation. This greatly sensitized the cells to doxorubicin such that cells were essentially gone by the time we measured apoptosis and necrosis. Consequently, our results show that inhibition of CypD is NOT a valid candidate for prostate cancer treatment. However, targeting of Fis1 and Parkin may have therapeutic value as they both sensitized prostate cancer cells to the necrotic effects of doxorubicin. Finally, we believe that Drp1 inhibition has the greatest therapeutic potential as even at baseline it inhibited cell proliferation, induced mitochondrial dysfunction, and promoted the greatest ROS production. A chemical inhibitor of Drp1 already exists (mDivi-1), and it will now be important to test whether this inhibitor can attenuate prostate cancer progression in animal models of this disease.

15. SUBJECT TERMS
Prostate cancer; Autophagy, Mitophagy; Mitochondria; Cyclophilin-D; Fis1; Drp1; Parkin; Cell death; Apoptosis; Necrosis

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INTRODUCTION

As a cancer cell progresses down the path of malignancy, it becomes more reliant upon autophagy as its main source of energy required for survival, proliferation, and migration. In particular, mitophagy - the selective removal of mitochondria - is critical for the continued survival of the cancer cell. Mitochondria from prostate cancer cells are more dysfunctional than those in normal cells in that they exhibit more mtDNA mutations and enhanced reactive oxygen species (ROS) production. Moreover, autophagy, and by inference mitophagy, enables prostate cancer cells to survive androgen deprivation. Therefore, mitophagy may represent a novel target for the prevention of prostate cancer progression and the development of anti-androgen resistance. *Thus, the current hypothesis is that the specific inhibition of mitophagy in prostate cancer cells will lead to cell death by 2 distinct mechanisms:* 1) by cutting off the cancer cell's main energy supply, and 2) by causing the accumulation of toxic dysfunctional mitochondria. Consequently, the *purpose* of this research is to test whether inhibition of mitophagy can lead to the death of prostate cancer cells. Key mediators of the mitophagic process, specifically Parkin, dynamin-related protein-1 (Drp1), fission-1 (Fis1), and cyclophilin-D (CypD), will be genetically disrupted by shRNA. The effects that mitophagy blockade has on mitochondrial function, ROS production and ultimately survival of normal prostate cells as well as several different prostate cancer cell lines, especially highly aggressive cells, will then be examined.

KEYWORDS

Prostate cancer; Autophagy, Mitophagy; Mitochondria; Cyclophilin-D; Fis1; Drp1; Parkin; Cell death; Apoptosis; Necrosis

OVERALL PROJECT SUMMARY

Knockdown of CypD, Fis1, Drp1, and Parkin in prostate cancer cell lines.

As detailed in the last progress report this initial step proved problematic as none of the shRNA-containing plasmids we obtained were able to knockdown any of the proteins in any of the cell lines (despite a lot of trouble shooting). However, when we switched to simple siRNAs, which were easier to transfect into cells, we have achieved significant knockdown of all 4 proteins (CypD, Fis1, Drp1, and Parkin) in the DU145, LNCaP and PC3 prostate cancer cell lines. As an example, **Figure 1A** shows the efficient knockdown of CypD, Fis1, Drp1 and Parkin in the DU145 cells. **Figure 1B** shows the level of knockdown for each protein in the DU145, LNCaP and PC3 prostate cancer cell lines. In all cases we achieved a significant reduction in protein levels ranging from ~20-50%. The one exception was Parkin levels in the PC3 cells. These cells appear to express low Parkin levels at baseline such that we only ever obtained a faint band by Western blotting. Although this band was reduced/disappeared with the Parkin siRNA, we were outside the linear range of detection and therefore we could not obtain an accurate assessment of the degree of knockdown. However, the fact that the siRNA was effective in the other 2 cancer cell lines and that we did see a phenotype leads us to believe that we achieved a sufficient Parkin knockdown in the PC3 cells.

The effects of CypD, Fis1, Drp1, and Parkin siRNAs on mitochondrial function.

Having now established effective siRNAs against each protein in the different cell lines, we then began to assess the effect of their knockdown on mitochondrial function. We first assessed the effects of the depletion of each protein on cellular ATP levels.

Somewhat to our surprise we did not see any major changes in ATP concentrations in response to the knockdown of any protein in any cell line (**Figure 2**). At first glance this would suggest that we are not greatly affecting mitochondrial function. However, most cells in culture primarily rely on glycolysis for their energy requirements, and this is especially true for cancer cells. Therefore any changes at the mitochondrial level would not have that great an effect on total ATP levels inside the cell.

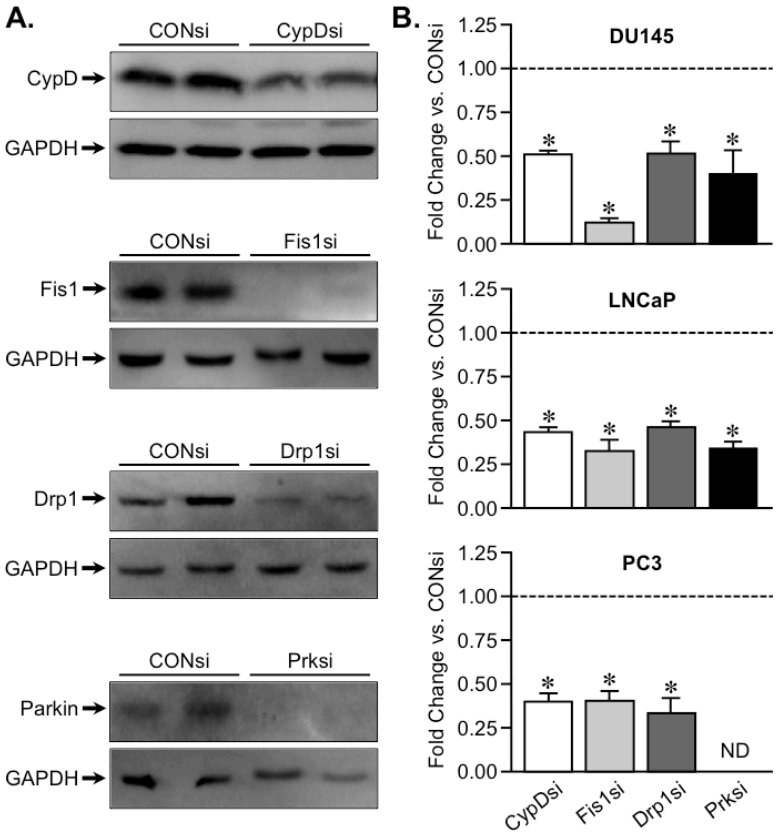

Figure 1. Effectiveness of siRNA knockdown of CypD, Fis1, Drp1, and Parkin in prostate cancer cells. (**A**) DU145 cells were transfected with either a control siRNA (CONsi) or specific siRNAs targeting Cypd, Fis1, Drp1, or Parkin. After 48 hrs the cells were harvested, lysed, and then subjected to immunoblotting for the respective protein. GAPDH was used as loading control. (**B**). Quantification of the degree of knockdown for each protein relative to control siRNA in the DU145, LNCaP, and PC3 prostate cancer cell lines. *P<0.05 vs.CONsi. ND, not determined.

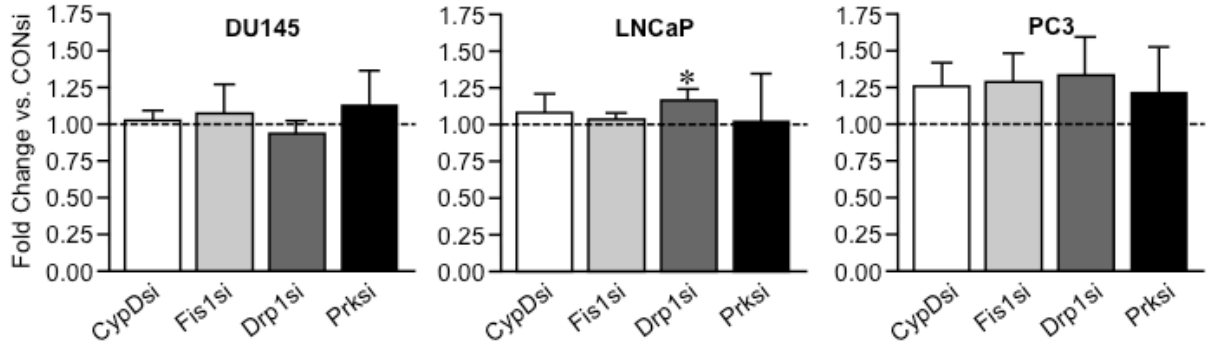

Figure 2. Effect of siRNA knockdown of CypD, Fis1, Drp1, and Parkin on total ATP levels in prostate cancer cells. (**A**) DU145, LNCaP, and PC3 cells were transfected with either a control siRNA (CONsi) or specific siRNAs targeting Cypd, Fis1, Drp1, or Parkin. After 48 hrs the cells were harvested, lysed, and ATP levels measured using a luminescence-based assay. Levels were then normalized to total protein. *P<0.05 vs.CONsi.

We next assessed whether knockdown of CypD, Fis1, Drp1, and/or Parkin had any major effects on the expression levels of key components of the 5 complexes that make up the electron transport chain. We used an antibody cocktail from Abcam that

contains 5 antibodies against proteins in each of the 5 respiratory complexes, thus allowing simultaneous visualization of each protein on the same gel. As an example, **Figure 3** shows expression of each of these proteins in LNCaP cells transfected with control, CypD, Fis1, Drp1, and Parkin siRNAs. When corrected for loading (using GAPDH) we did not find much in the way of significant changes in the majority of the complexes in response to the different siRNAs in either the DU145, LNCaP, or PC3 cell lines. The exceptions were a significant reduction in the complex-I protein in response to both Parkin and Drp1 siRNAs in the DU145 and PC3 cells. This tendency was also seen in the LNCaP cells. However, it did not reach significance due to variability (for example it is unchanged in **Figure 3**). There was also a trend for the CypD siRNA to increase complex-II expression in all 3 of the cancer cell lines, but again this was not significant.

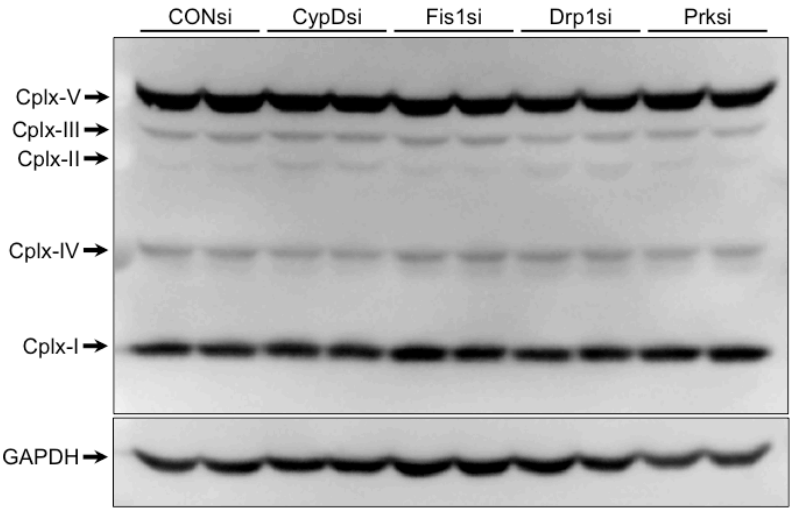

Figure 3. Effect of knockdown of CypD, Fis1, Drp1, and Parkin on mitochondrial complexes in LNCaP prostate cancer cells. LNCaP cells were transfected with either a control siRNA (CONsi) or specific siRNAs targeting Cypd, Fis1, Drp1, or Parkin for 48 hrs. The cells were then harvested, lysed, and subjected to immunoblotting for respiratory complex (Cplx) proteins. The specific proteins are NDUFB8 (I), SDHB (II), UQCRC2 (III), COX-II (IV), and ATP5A (V). GAPDH was used as loading control.

We next determined the mitochondrial membrane potential in the transfected DU145, LNCaP, and PC3 cells using the potentiometric dye TMRE. The higher the potential the more dye is taken up, and the higher the resultant fluorescence. Reduced mitochondrial membrane potential can be indicative of mitochondrial dysfunction and damage (although not always). Knockdown of CypD actually increased the level of TMRE fluorescence in all of the cell lines (**Figure 4**). This indicates that removal of CypD may actually be beneficial for the cancer cells' mitochondria, consistent with CypD's role as a critical regulator of the mitochondrial permeability transition pore, opening of which causes mitochondrial dysfunction, rather than of mitophagy.

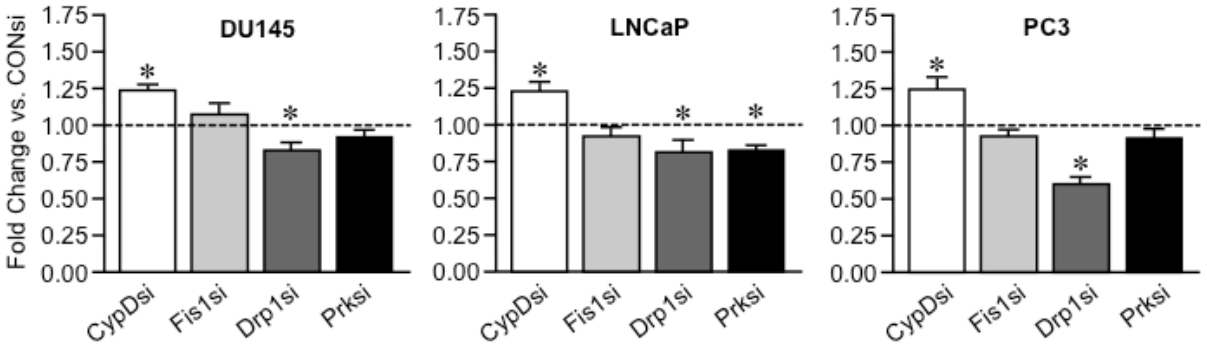

Figure 4. Effect of siRNA knockdown of CypD, Fis1, Drp1, and Parkin on mitochondrial potential in prostate cancer cells. DU145, LNCaP, and PC3 cells were transfected with either a control siRNA (CONsi) or specific siRNAs targeting Cypd, Fis1, Drp1, or Parkin. After 48 hrs the cells were stained with TMRE. The levels of fluorescence were then quantified using NIH ImageJ software. *P<0.05 vs.CONsi.

Knockdown of Fis1, which mediates the mitochondrial fragmentation required for mitophagy, had no effect on mitochondrial potential (**Figure 4**). This is in stark contrast to the depletion of Drp1, another mitochondrial fragmentation protein, which depressed mitochondrial potential in all 3 prostate cancer cell lines (**Figure 4**). Parkin knockdown yielded mixed results with no significant reduction in mitochondrial potential seen in the DU145 and PC3 cells, and a small reduction observed in the LNCaP cells. This was surprising given that Parkin knockdown depleted complex-I, which would be expected to reduce membrane potential. However, the cells are probably bypassing complex-I and utilizing complex-II instead as the jumping on point for oxidative phosphorylation (Indeed, increased complex-II, although not significant, may explain the increases in potential in response to CypD siRNA). Using this argument, we would have expected the same result with Drp1 siRNA. However, we believe that the reduction of complex-I combined with inhibited mitochondrial fragmentation leads to the reduction in potential and that fusion (Fis1 siRNA) or complex-I deficiency (Parkin siRNA) are not sufficient to induce the deficit alone.

Mitochondria dysfunction is often accompanied by an increase in mitochondrially-derived reactive oxygen species (ROS). In accordance with our hypothesis a build up of dysfunctional mitochondria due to decreased mitophagy would be expected to increase ROS levels. To this end we measured ROS production using the ROS-sensitive fluorescent dye DCF. We initially planned to use the mitochondrially-targeted indicator MitoSox. However, although achieving mitochondrial loading with this agent, the signal was not robust enough. We therefore changed to DCF, which gives a stronger signal. Analysis of the fluorescent images revealed that CypD depletion did not greatly affect ROS production in the LNCaP and PC3 cells and actually decreased production in the DU145s (**Figure 5**). Again, this is consistent with inhibition of the mitochondrial permeability transition pore, and fits with the improved mitochondrial potential in these cells. Depletion of Parkin had no major effect on ROS production in any of the cell lines **(Figure 5**). In contrast, both Fis1 and Drp1 knockdown increased ROS in both the LNCaP and PC3 lines, with Drp1 depletion even tending to increase ROS production in the DU145 cells as well (**Figure 5**). Given that both Fis1 and Drp1 had similar effects, our conclusion is that this ROS production is a byproduct of inhibition of mitochondrial fragmentation rather than inhibition of mitophagy *per se*.

Finally, we were going to measure mtDNA as an index of mitochondrial number. However, we then

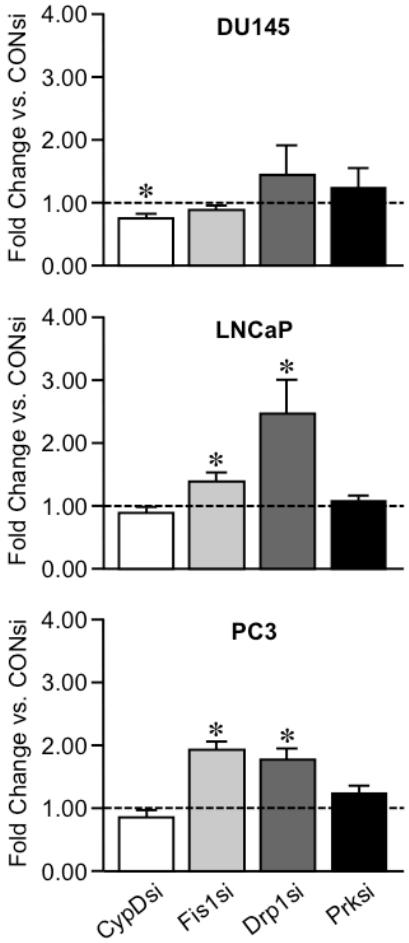

Figure 5. Effect of siRNA knockdown of CypD, Fis1, Drp1, and Parkin on ROS levels in prostate cancer cells. DU145, LNCaP, and PC3 cells were transfected with either a control siRNA (CONsi) or specific siRNAs targeting Cypd, Fis1, Drp1, or Parkin. After 48 hrs the cells were stained with DCF. The levels of fluorescence were then quantified using NIH ImageJ software. **P*<0.05 vs.CONsi.

realized that the Complex-IV protein, COX-II, used in our respiratory complex blots is derived from the mtDNA and therefore is an index of mitochondrial number itself. As mentioned above we did not see any changes in COX-II expression in response to any siRNA in any of the cell lines. This was at first a concern as we expected to see an increase in COX-II expressionas we were hopefully inhibiting mitophagy, thereby increasing the number of mitochondria per cell. However, as detailed in the next section we believe this number is stagnant due to a compensatory increase in general autophagy to combat the inhibition of the more specialized mitophagy.

The effects of CypD, Fis1, Drp1, and Parkin siRNAs on autophagy and mitophagy.

The next part of the study was to examine general markers of autophagy and then assess mitophagy itself. Two key components of the autophagic signaling system are Beclin which triggers expansion of the isolation membrane to engulf cell constituents and LC3 which is incorporated into the forming autophagosome. We therefore examined expression levels in the siRNA transfected DU145, LNCaP, and PC3 cells (an example of the Western blots is shown for PC3 cells in **Figure 6A**). To our surprise, all 4 siRNAs (CypD, Fis1, Drp1, and Parkin) elevated Beclin levels in all 3 prostate cancer cell lines (**Figure 6B**), suggesting a compensatory upregulation of Beclin in response to inhibition of mitophagy. Interestingly however, a concomitant upregulation of LC3 was only seen in the Fis1, Drp1, and Parkin depleted cells (**Figure 6C**), especially in the LNCaP and PC3 cells, indicating that the compensatory signal is then inhibited distal to Beclin in the CypD siRNA-transfected cells. We also measured the levels of upstream regulators of autophagy, namely mTOR (which inhibits autophagy), and AMPK

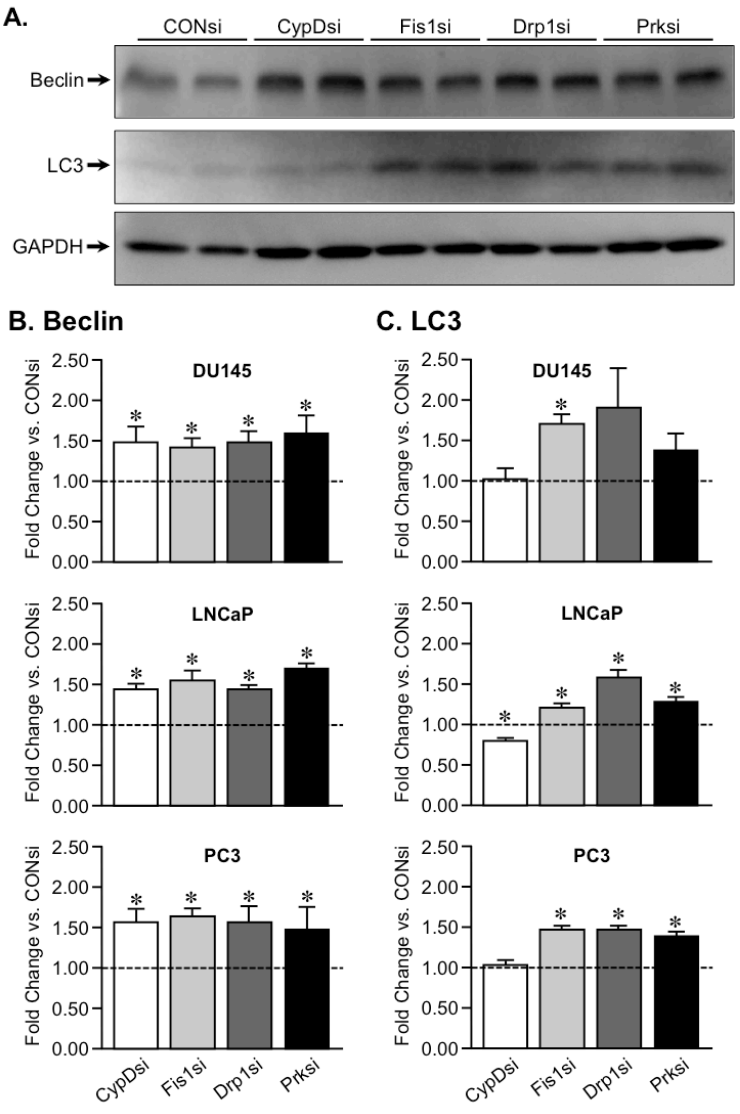

Figure 6. Effect of siRNA knockdown of CypD, Fis1, Drp1, and Parkin on Beclin and LC3 levels in prostate cancer cells. (**A**) PC3 cells were transfected with either a control siRNA (CONsi) or specific siRNAs targeting Cypd, Fis1, Drp1, or Parkin. After 48 hrs the cells were harvested, lysed, and immunoblotted Beclin and LC3. GAPDH was used as loading control. (**B,C**). Quantification of the expression of Beclin (**B**) and LC3 (**C**) relative to control siRNA in the DU145, LNCaP, and PC3 prostate cancer cell lines. **P*<0.05 vs.CONsi.

(which inhibits mTOR, and therefore stimulates autophagy). However, we saw no significant changes in either of these proteins in the various transfected cells (data not shown).

While increases in Beclin and LC3 are suggestive of autophagy they are not an absolute indicator. For example, general levels of LC3 maybe up but whether they are being incorporated into autophagosomes is another matter. To address this we conducted parallel studies where we infected the transfected cells with a GFP-LC3 marker. The majority of the GFP signal is diffuse through the cytoplasm. However, as the GFP-LC3 gets incorporated into the autophagosome it forms bright fluorescent puncta (an example is shown in **Figure 7**). Therefore these puncta are an index of autophagy. In terms of absolute numbers, the number of LC3 puncta-containing cells at baseline was in the order of PC3 > LNCaP > DU145.

We then counted the number of puncta-containing cells in each of the groups. Remarkably, we observed specific increases in LC3 puncta-containing cells that mirrored those seen with increases in ROS production (see **Figure 5**). Specifically, the numbers of cells with GFP-LC3 puncta were elevated in the Fis1 and Drp1-depleted LNCaP and PC3 cells, and in the Drp1-depleted DU145 cells (**Figure 8**). This suggests that it is the generation of ROS that is responsible for the increase in autophagic cells. This is consistent with several reports indicating that increased ROS can induce autophagy in variety of human cancer cell lines, including prostate. Taken together with the Beclin and LC3 expression data, this indicates that inhibition of mitophagy and/or mitochondrial fragmentation induces a large compensatory increase in general autophagy. There was a tendency for Parkin siRNA to increase the number of puncta-containing cells in all 3 lines but this did not reach statistical significance, possibly due to the lack of ROS changes in these groups of cells.

The final part of this series of experiments was to measure mitophagy itself. To achieve this we loaded the transfected cells with Lysotracker-Red to stain the lysosomes and Mitotracker-Green to label the mitochondria. Co-localization of the 2 dyes would

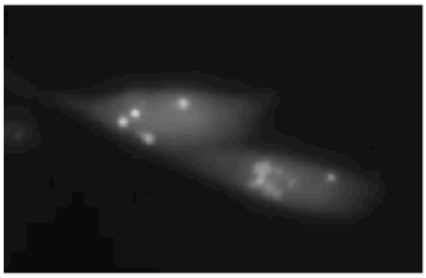

Figure 7. Example of GFP-LC3 puncta. Control PC3 cells were infected for 24 hrs with an adenovirus encoding for GFP-tagged LC3.

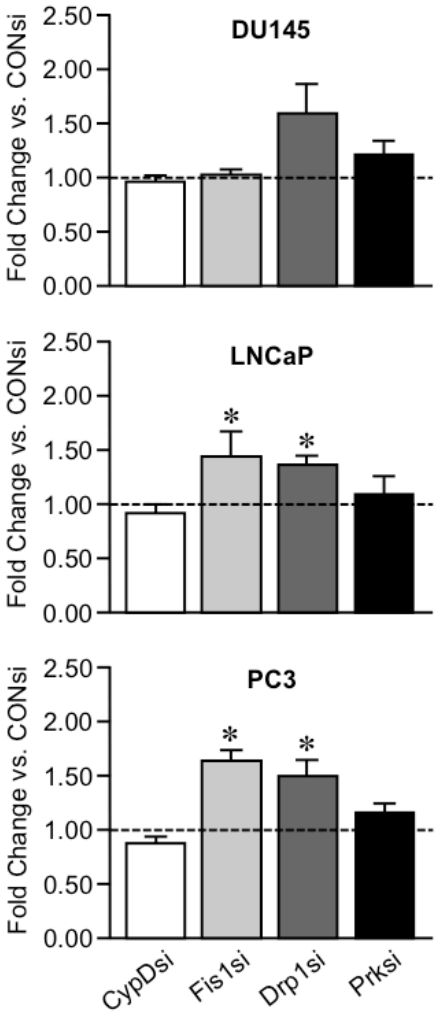

Figure 8. Effect of siRNA knockdown of CypD, Fis1, Drp1, and Parkin on GFP – LC3 puncta in prostate cancer cells. DU145, LNCaP, and PC3 cells were transfected with control siRNA (CONsi) or siRNAs targeting Cypd, Fis1, Drp1, or Parkin. After 48 hrs the cells were infected for another 24 hrs with the GFP-LC3 virus and the number of puncta-containing cells quantified. *P<0.05 vs.CONsi.

indicate that mitochondria were inside the lysosomal compartment and had therefore undergone mitophagy. A decrease in co-localization would indicate decreased mitophagy whereas in increase would suggest the opposite. We assessed the level of co-localization using NIH ImageJ software, which measures overlapping signals independent of the signal intensity. The output is a Pearson's Coefficient with the higher the Coefficient the higher the co-localization. The results obtained were similar to thos to LC3 expression suggestion a link between the two. Knockdown of CypD significantly reduced he Person's Coefficient in the LNCaP and PC3 cells suggesting decreased mitophagy (**Figure 9**). However, opposite to our expectation, we actually saw an increase in mitochondria-lysosomal co-localization in response to Fis1, Drp1, and Parkin knockdown in all cell lines (the one exception being Fis1 siRNA in the PC3 cells; **Figure 9**). This was counterintuitive, as we would have expected decreases due to an inhibition of mitophagy. However, it would appear that the compensatory increase in

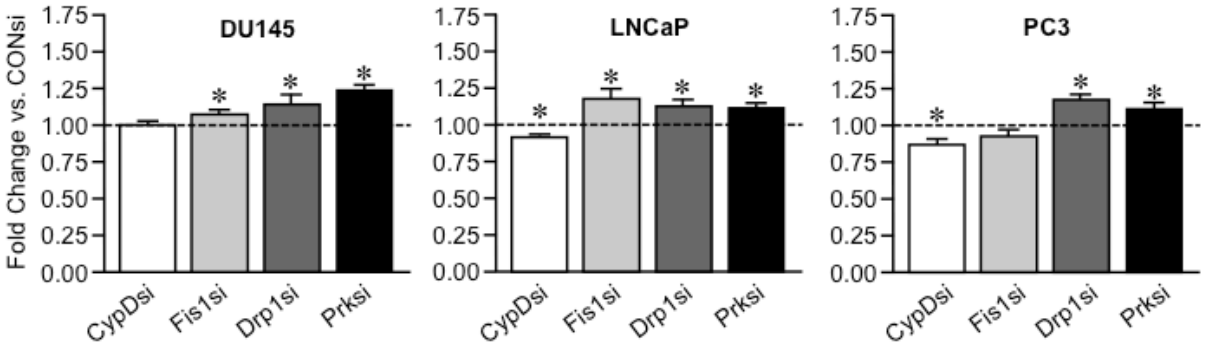

Figure 9. Effect of siRNA knockdown of CypD, Fis1, Drp1, and Parkin on mitophagy in prostate cancer cells. DU145, LNCaP, and PC3 cells were transfected with either a control siRNA (CONsi) or specific siRNAs targeting Cypd, Fis1, Drp1, or Parkin. After 48 hrs the cells were stained with lysotracker-Red and Mitotracker-Green. Fluorescence co-localization was then quantified using NIH ImageJ software. **P*<0.05 vs.CONsi.

general autophagy is sufficient to overcome this inhibition and even surpass the original levels of mitochondrial digestion. One question of course is why does inhibition of mitophagy by CypD knockdown not induce a compensatory response, whereas the others do? This maybe related to the fact that CypD knockdown if anything reduces ROS production and increases membrane potential, thus reducing triggers for autophagy. We are currently going back through images and analyzing them on an individual cell basis. This is laborious and painstaking but may provide more mechanistic insight as to what is going on.

The effects of CypD, Fis1, Drp1, and Parkin siRNAs on apoptosis and necrosis. Ultimately the goal of the study was to see if inhibition of mitophagy could exacerbate cell death in these prostate cancer cells either at baseline or in response to a chemotherapeutic agent. Our original agent of choice was paclitaxel. However, when we started experiments with this compound we found that it detached all of the cells from the plate well before the induction of cell death, making it impossible to analyze them *in situ*. To this end we switched to another agent used for prostate cancer treatment, doxorubicin. Here the cells were better retained on the plate thereby allowing us to assess apoptosis and necrosis. The one exception was the Drp1 siRNA transfected cells, which exhibited considerable detachment – we believe this is an important finding, which will be discussed in the context of new data in the next section.

From a pure “death responsiveness” perspective we found an order of LNCaP >> DU145 >> PC3, with the LNCaP cells being very sensitive to doxorubicin as opposed to the PC3 cells which were highly resistant.

We first examined the levels of apoptosis in the transfected cell lines by means of TUNEL staining, as well as Western analyzes of cleaved caspase-3 and PARP1, 2 hallmarks of apoptotic execution. The DU145 cells were the first to be tested. In the control siRNA transfected cells, increasing concentrations of doxorubicin (0.5, 1, and 2μM) for 24 hrs induced a dose-dependent increase in the cleavage of caspase-3 and PARP1, as well as in TUNEL staining (**Figure 10**). Knockdown of CypD had no significant effect on the levels of any of the apoptotic indices in response to doxorubicin. In contrast Fis1 induced mild reductions in the cleavage of caspase-3 and PARP and a small but significant reduction in the levels of TUNEL staining (**Figure 10**), suggesting a mild protective effect. Opposite and dichotomous effects were observed for the Drp1 and Parkin siRNAs. Drp1 depletion had no effect on caspase-3 and PARP cleavge yet reduced the levels of TUNEL staining (**Figure 10**). In contrast Parkin depletion significantly reduced cleavage of the apoptotic substrates yet had no real effect on TUNEL staining (**Figure 10**). With Drp1, as mentioned above we are losing cells from the plate after treatment, thus the TUNEL staining is probably underrepresenting the levels of apoptosis as it only detects cells still adherent to the dish. When we do the Westerns we collect both the adherent and the floating cells, which may give a more accurate indication of apoptosis. The effects of Parkin depletion are harder to explain. What might be happening is that the cells initially undergo a rapid apoptosis (and therefore stain positive for TUNEL), but then switch to necrosis, which leads to the release and loss of the caspase-3 and PARP1 products. This would imply that the cell death process is accelerated in these cells.

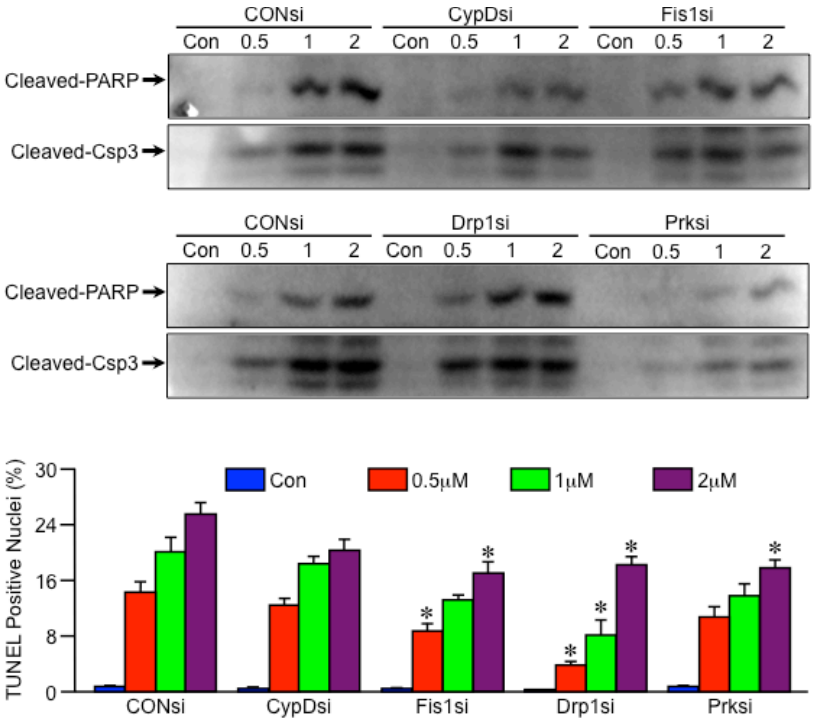

Figure 10. Effect CypD, Fis1, Drp1, and Parkin siRNAs on doxorubicin-induced apoptosis in DU145 prostate cancer cells. Upper panels, DU145 cells were transfected with a control siRNA (CONsi) or specific siRNAs targeting Cypd, Fis1, Drp1, or Parkin for 48 hrs and then treated with increasing concentrations of doxorubicin. The cells were then subjected to immunoblotting for cleaved caspase-3 and PARP. Lower panel, TUNEL staining in the siRNA transfected and doxorubicin-treated cells. *P<0.05 vs. corresponding dose in the CONsi group.

We then repeated these experiments in the LNCaP cells. As mentioned above these cells were much more sensitive to doxorubicin. In fact they were an order

of magnitude more sensitive than the DU145 cells, and we therefore reduced the doxorubicin concentrations to 0.05, 0.1, and 0.2μM. Similar to the DU145 cells, knockdown of CypD had no discernable effect on cleaved caspase-3, cleaved PARP, and TUNEL staining, when compared to control siRNA-transfected cells, in response to the increasing amounts of doxorubicin, (**Figure 11**). Both Fis1 and Parkin depletion had similar effects in that TUNEL positivity was unaltered but the cleavage of both caspase-3 and PARP was markedly reduced (**Figure 11**). Again, we think that this is due to an accelerated death program such that the cells undergo apoptosis and then switch to a necrotic phenotype (as evidenced when we look at necrosis below).

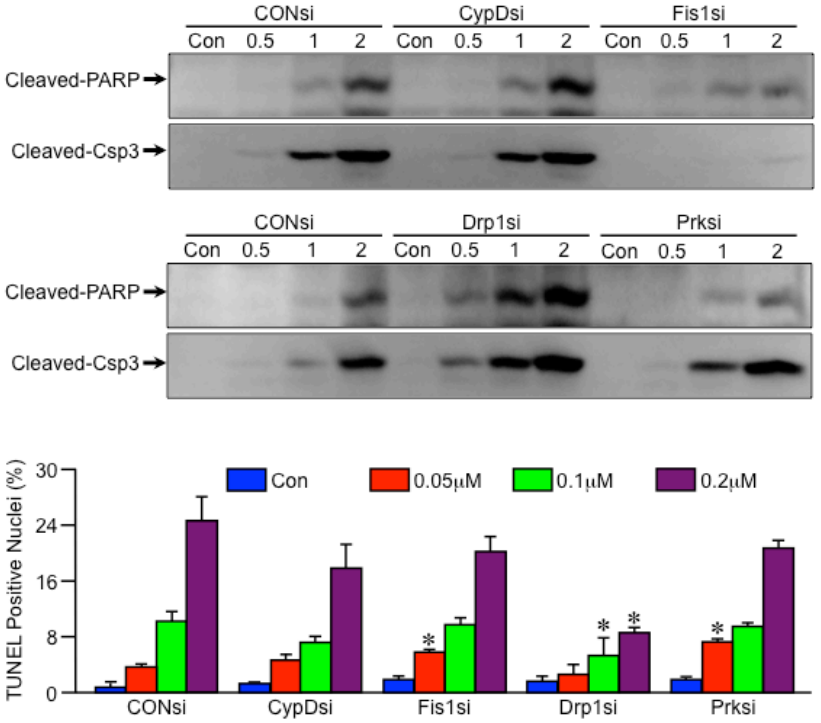

Figure 11. Effect CypD, Fis1, Drp1, and Parkin siRNAs on doxorubicin-induced apoptosis in LNCaP prostate cancer cells. Upper panels, LNCaP cells were transfected with a control siRNA (CONsi) or specific siRNAs targeting Cypd, Fis1, Drp1, or Parkin for 48 hrs and then treated with increasing concentrations of doxorubicin. The cells were then subjected to immunoblotting for cleaved caspase-3 and PARP. Lower panel, TUNEL staining in the siRNA transfected and doxorubicin-treated cells. **P*<0.05 vs. corresponding dose in the CONsi group.

Similar to the DU145s, Drp1 knockdown exhibited a dichotomous response with if anything enhanced the cleavage of the apoptotic proteins but reduced TUNEL positivity (**Figure 11**). In this case there were very few cells left attached to the plate after doxorubicin, even at the lower concentration, again resulting in an under-representation of TUNEL positive cells.

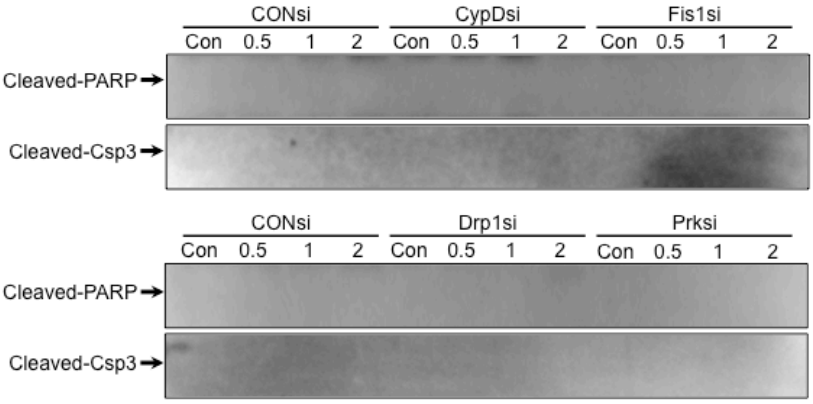

Figure 12. Effect CypD, Fis1, Drp1, and Parkin siRNAs on doxorubicin-induced apoptosis in PC3 prostate cancer cells. PC3 cells were transfected with a control siRNA (CONsi) or specific siRNAs targeting Cypd, Fis1, Drp1, or Parkin for 48 hrs and then treated with increasing concentrations of doxorubicin. The cells were then subjected to immunoblotting for cleaved caspase-3 and PARP.

Regarding apoptosis we lastly examined the PC3 cells. These cells were profoundly resistant to doxorubicin-induced apoptosis to the point that we could not detect any cleavage of either caspase-

3 or PARP by Western blotting at all (**Figure 12**). Consistent with these data we found that levels of TUNEL positivity were very low (less than 1%) and did not significantly increase with doxorubicin treatment even up to concentrations as high as 10μM, regardless of which siRNA was transfected (data not shown).

Lastly we examined necrotic cell death in the various cells groups. To do this we utilized the vital dye Sytox Green. Sytox is membrane impermeable and therefore will not enter healthy living cells. However, when a cell has undergone membrane rupture, a hallmark of necrosis, Sytox enters the cell where it binds the DNA in the nucleus and fluoresces. In all 3 cell lines, doxorubicin induced a dose-dependent increase in necrosis in the control siRNA group (albeit at very low levels in the PC3 cells, **Figure 13**). This was significantly reduced in the CypD siRNA-transfected cells (**Figure 13**).

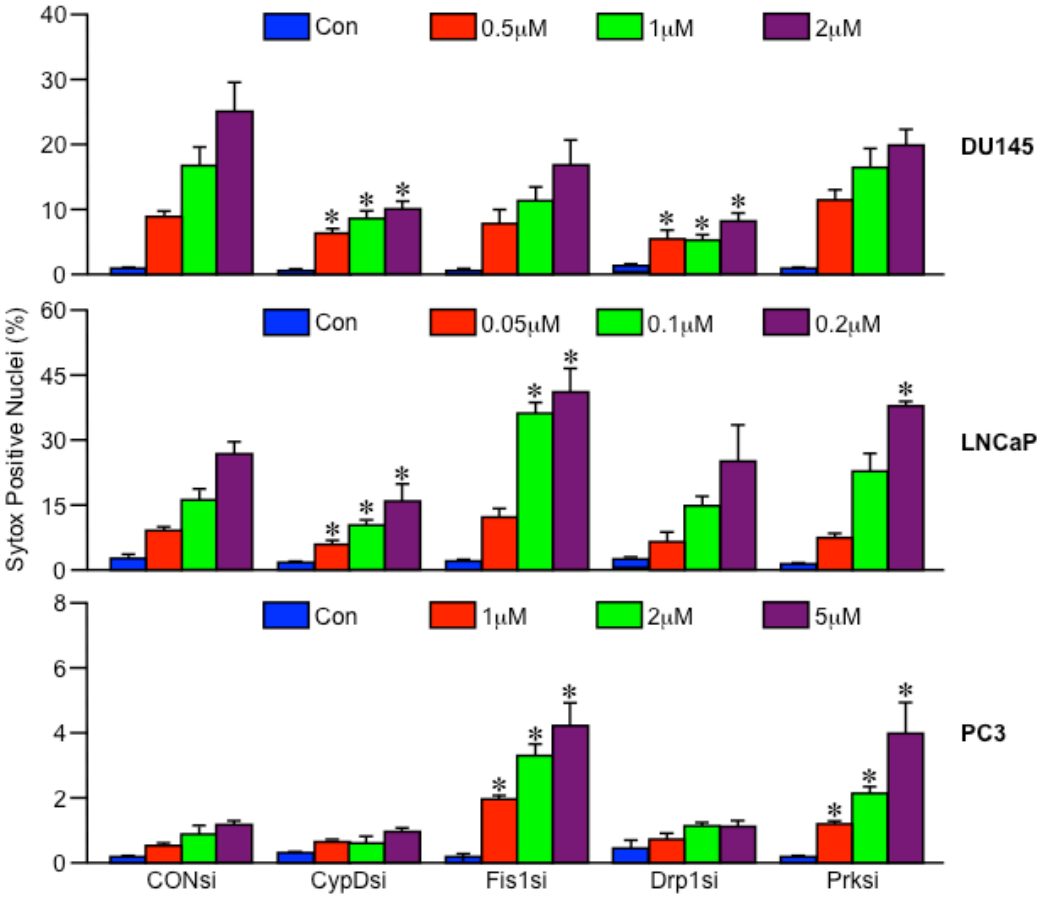

Figure 13. Effect CypD, Fis1, Drp1, and Parkin siRNAs on doxorubicin-induced necrosis in the prostate cancer cells. DU145 (upper panel) LNCaP (middle panel), and PC3 (lower panel) cells were transfected with a control siRNA (CONsi) or specific siRNAs targeting Cypd, Fis1, Drp1, or Parkin for 48 hrs and then treated with increasing concentrations of doxorubicin. The cells were then stained with Sytox Green (to stain necrotic cells) and bis-benzimide (to stain all cells) and quantified. *P<0.05 vs. corresponding dose in the CONsi group.

Given that doxorubicin is thought to induce necrosis through opening of the mitochondrial permeability transition pore, this effect is not surprising as the pore is desensitized when CypD levels are low. Reduction in Fis1 or Parkin levels did not affect the levels of necrosis in the Du145 cells (**Figure 13**). However, both siRNAs significantly increased necrotic death in the LNCaP and PC3 cells (**Figure 13**). In the case of the LNCaP cells, this fits with our notion that cell death is accelerated in these

cells when Fis1 and Parkin are reduced. Drp1 depletion for the most part had no effect on the number of Sytox positive cells and was even reduced in the DU145s (**Figure 13**). However, similar to the TUNEL experiments, we had very few cells left attached, especially in the LNCaP group, so that the overall number of positive cells is underestimated.

As a final estimate of necrosis we planned to measure release of HMGB1 into the media. When we just used the media directly, it was too dilute to get a usable Western blot signal. However, when we concentrated the media the signal from the serum proteins in the media became so overwhelming they masked the HMGB1. Thus were not able to conduct these particular experiments, but we are continuing to trouble shoot to see if we can remove the serum proteins.

The effects of CypD, Fis1, Drp1, and Parkin siRNAs on cell proliferation.

As we were conducting these experiments we kept observing that knockdown of CypD appeared to increase the number of cells in the plate. In contrast knockdown of Drp1 seemed to reduce cell numbers. In fact Drp1 depletion caused all of the cancer cells to appear more rounded, i.e., less elongated and there were even some floating cells, especially in the "sensitive" LNCaP cells. To get a better handle on this we measured cell numbers by staining for nuclei using bis-benzimide in the different cell lines transfected with the various siRNAs. As can be seen in **Figure 14** there was a marked increase in DU145, LNCaP and PC3 cell numbers when CypD was knocked down. In contrast, Drp1 significantly reduced cell numbers in all of the cell lines (**Figure 14**). Fis1 and Parkin siRNAs were without effect. These changes correspond to those seen with mitochondrial membrane potential (see **Figure 4**), where CypD knockdown increased potential and Drp1 knockdown decreased potential. In fact Drp1 knockdown had the most detrimental effects on the cells, reducing mitochondrial potential, increasing ROS production and reducing cell number, and apparently viability, even at baseline. As mentioned above, we believe that the majority of cell death occurs early in the Drp1-depleted cells such that at 24 hrs we have missed the "window" and the cells have detached. Future experiments will be aimed at finding out at which time point within the 24 hr treatment the maximum amount of death occurs.

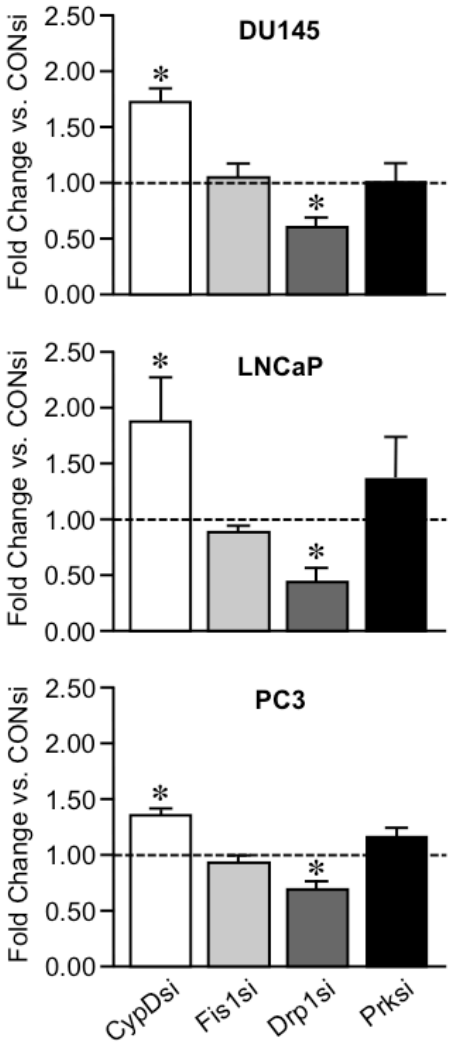

Figure 14. Effect of siRNA knockdown of CypD, Fis1, Drp1, and Parkin on cell number in prostate cancer cells. DU145, LNCaP, and PC3 cells were transfected with control siRNA (CONsi) or siRNAs targeting Cypd, Fis1, Drp1, or Parkin. After 48 hrs the cells were stained with bis-benzimide and number counted. **P*<0.05 vs.CONsi.

KEY RESEARCH ACCOMPLISHMENTS

- Identified and characterized siRNAs that efficiently knocked down CypD, Fis1, Drp1, and Parkin in DU145, LNCaP, and PC3 prostate cancer cells.
- Found that on the whole, inhibition of mitophagy causes a compensatory upregulation of general autophagy and that this appears to be a direct result of increased ROS production.
- Found that CypD depletion beneficially, rather than detrimentally, affects mitochondrial function and ROS production and is protective against doxorubicin-induced cell death.
- Demonstrated that Fis1 and Parkin knockdown are sufficient to sensitize LNCaP and PC3 cells to the necrotic effects of doxorubicin and therefore may be targets for therapy.
- Found that Drp1 knockdown was sufficient to impair mitochondrial function, induce ROS production and reduce cell proliferation. This greatly sensitized the cells to doxorubicin such that cells were essentially gone by the time we measured apoptosis and necrosis.

CONCLUSION

Our work has shown that inhibition of the various components of mitophagy in prostate cancer cells can have differing effects on the mitochondrial function, autophagic rate, and sensitivity to chemotherapeutics. However, we believe that it has revealed several potential targets for therapeutic agents, most likely as adjunct therapies to sensitize prostate cancer cells to chemotherapy. Our work has convincingly demonstrated that inhibition of CypD is NOT a valid candidate for intervention as depletion of CypD improved mitochondrial function, increased proliferation and protected against cell death. In contrast, targeting of Fis1 and Parkin may have therapeutic value as they both sensitized prostate cancer cells to the necrotic effects of doxorubicin, despite having differing effects on autophagy and ROS production. Indeed induction of necrosis may be more preferable to apoptosis as necrosis will induce an inflammatory/immune response that will further enhance cancer cell killing. Finally, we believe that Drp1 inhibition has the greatest therapeutic potential as even at baseline it inhibited cell proliferation, induced mitochondrial dysfunction, and promoted the greatest ROS production. Thus Drp1 inhibition may even have direct benefits irrespective of chemotherapy. Given that a chemical inhibitor of Drp1 already exists (mDivi-1), it will be important to test whether this inhibitor can attenuate prostate cancer progression in animal models of this disease.

PUBLICATIONS, ABSTRACTS, AND PRESENTATIONS

Nothing to report.

INVENTIONS, PATENTS, AND LICENSES

Nothing to report.

REPORTABLE OUTCOMES

Nothing to report.

OTHER ACHEIVEMENTS

Nothing to report.